Journal of Chromatography, 230 (1982) 175—180
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1222

Note

Liquid chromatographic separation and quantitation of 2-amino-1,3,4-thiadiazole (NSC-4728) from human and murine serum

CYNTHIA C. ACKERLY, ROBERT A. NEWMAN*, CARL MYERS and JOHN J. McCORMACK

*Vermont Regional Cancer Center and *Departments of Pharmacology and Medicine, College of Medicine, Given Building, University of Vermont, Burlington, VT 05405 (U.S.A.)*

(First received August 28th, 1981; revised manuscript received January 13th, 1982)

The compound 2-amino-1,3,4-thiadiazole (ATDA; NSC-4728) is currently undergoing Phase I clinical trials as an experimental antitumor agent at the Vermont Regional Cancer Center (Fig. 1). Previous clinical investigational trials with ATDA have been limited due to the drug-mediated production of stomatitis and hyperuricemia [1, 2]. These adverse side effects can now be adequately controlled by the administration of nicotinamide which abrogates all effects of ATDA and allopurinol which blocks the excessive formation of uric acid [2, 3].

Pharmacokinetic studies of ATDA have demonstrated half-lives of 2.9 h in mice [4] and 10 h in dogs [5]. Similar studies had not yet been performed in humans. Therefore, in order to evaluate the pharmacokinetics of ATDA, a procedure for the routine quantitation of drug from serum samples was required. A combined system of thin-layer (TLC) and high-performance liquid chromatography (HPLC) was developed to enable the user to quantitate not only ATDA but also allopurinol and nicotinamide.

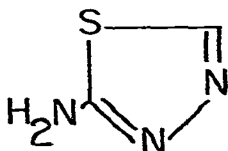

Fig. 1. Structure of 2-amino-1,3,4-thiadiazole (ATDA).

0378-4347/82/0000—0000/$02.75 © 1982 Elsevier Scientific Publishing Company

EXPERIMENTAL

Standards and reagents

HPLC grade water, 2-propanol, acetonitrile, and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). ATDA (NSC-4728) was obtained from the Investigational Drug Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD, U.S.A.). Allopurinol (Zyloprim®) is a product of Burroughs-Wellcome (Research Triangle, NC, U.S.A.). Nicotinamide and uric acid were obtained from Sigma (St. Louis, MO, U.S.A.). Paired-ion chromatographic reagent (Pic B-8, 5 m*M* 1-octanesulfonic acid) was purchased from Waters Assoc. (Milford, MA, U.S.A.).

Stock solutions of ATDA, allopurinol, nicotinamide, and uric acid were prepared by dissolving the compounds in HPLC grade water. Further dilutions were then prepared as needed for injection.

Chromatographic apparatus

A Spectra Physics Model 8000 microprocessor-controlled high-performance liquid chromatograph equipped with a data system was used. The chromatograph was equipped with a Schoeffel Model 770 variable-wavelength UV detector set at 254 nm. The column (30 cm × 3.9 mm I.D.) was a 10-μm particle size reversed-phase C_{18} μBondapak (Waters Assoc.). A guard column (7 cm × 0.2 mm I.D.), packed with Co:Pell ODS, 30—38 μm particle size (Whatman, Clifton, NJ, U.S.A.) was installed to protect the main column. Samples were injected onto the column through a 100-μl loop using a manual injector. Avicel-F (250-μm particle size) TLC plates were obtained from Analtech (Newark, DE, U.S.A.).

Drug administration and serum collection

Human. ATDA was administered to patients with cancer unresponsive to conventional therapy after written informed consent was obtained. Drug was administered over a 30-sec period by injection into a running intravenous line containing normal saline. Doses up to 200 mg/m^2 of body surface area (4 to 5 patients per dose) have been examined so far in this Phase I trial. Approximately 10 ml of whole blood were obtained from the arm contralateral to that used for drug injection. Blood samples were centrifuged (600 *g*, 10 min) and the resulting serum was stored up to 1 week at −20°C.

Mouse. Female CD-1 mice (18—22 g) were injected intravenously (tail vein) with 300 mg/m^2 of body surface (100 mg/kg). Blood samples were obtained by retro-orbital puncture.

Sample preparation and drug analysis

Human serum samples were prepared for TLC by addition of 9 volumes of acetonitrile. After centrifugation (600 *g*, 10 min at 5°C), the supernatant was collected and brought to dryness under vacuum with air purging at 37°C using a Fisher IMD sample concentrator (Fisher Scientific, Pittsburgh, PA, U.S.A.). The residue was reconstituted with 0.4 ml HPLC grade water. The samples (50-μl aliquots) were manually spotted onto the TLC plates along with the appropriate ATDA, nicotinamide, allopurinol, and uric acid standards. Murine

serum contained sufficient ATDA concentration to permit spotting of 10 μl of serum directly onto the TLC plates.

TLC plates were developed at ambient room temperature in a closed chamber over a 5-h period using a solvent system of 2-propanol—water (70:30, v/v). This procedure resulted in R_F values of 0.42 for uric acid, 0.78 for allopurinol, 0.80 for ATDA and 0.92 for nicotinamide. UV (254 nm) absorbing spots, which corresponded to uric acid or nicotinamide standards, were scraped separately from the plate, eluted in 0.4 ml water, sonicated and analyzed individually with the HPLC system. The single spot containing both ATDA and allopurinol was likewise scraped from the plate, eluted in 0.4 ml water with sonication and injected onto the HPLC apparatus.

HPLC conditions include a mobile phase consisting of an isocratic system of water—methanol (99:1, v/v) and the paired-ion reagent 1-octanesulfonic acid (buffered to a pH of 3.0) at a final concentration of 5 m*M* with a flow-rate of 1.8 ml/min. The concentration of each of the four compounds was measured by UV absorption at 254 nm which was considered optimal for ATDA. Quantitative analysis was based on peak areas which were computed using a preset integration program in the software data system of the Spectra-Physics instrument.

RESULTS AND DISCUSSION

The initial sample preparation, which consists of deproteinization with acetonitrile and TLC, permits removal of interfering protein, concentration of the resulting supernatant and the separation of ATDA and allopurinol from uric acid as well as nicotinamide. HPLC injection of a water extract of the ATDA—allopurinol TLC spot provides complete separation and quantitation of these two drugs. Shown in Fig. 2 is a chromatogram demonstrating the separation of all four compounds. The use of this method, which was specifically designed for the analysis of ATDA, permits routine quantitation of this drug in biological fluids, such as serum, that contain ATDA levels as low as 400 ng/ml. Fig. 3 consists of two chromatograms: a demonstration of a serum blank obtained immediately prior to the administration of ATDA and the same patient's serum 5 min post ATDA administration.

A standard calibration curve for ATDA was prepared daily by plotting peak area against the concentration of injected drug. This relationship was linear over a one-hundred-fold concentration range (5—500 ng of injected drug) with an average correlation coefficient of 0.996 using the least squares regression method. The minimal detectable level of injected ATDA was 10 ng at a detector setting of 0.01 a.u.f.s. and a recorder attenuation of zero. The signal-to-noise ratio was five or greater under these conditions.

To determine the recovery and reproducibility of the method, 1-ml aliquots of serum were spiked with ATDA to yield drug concentrations of 25, 50, 100 and 200 μg/ml prior to the deproteinization step. These samples were compared to the peak areas from directly injected ATDA standards. An average recovery of 89.6 ± 1.3% was obtained at all drug concentrations, with an intra-assay coefficient of variation of less than 2.0% (n = 5). This procedure was used also as an external standard method of monitoring day-to-day percent

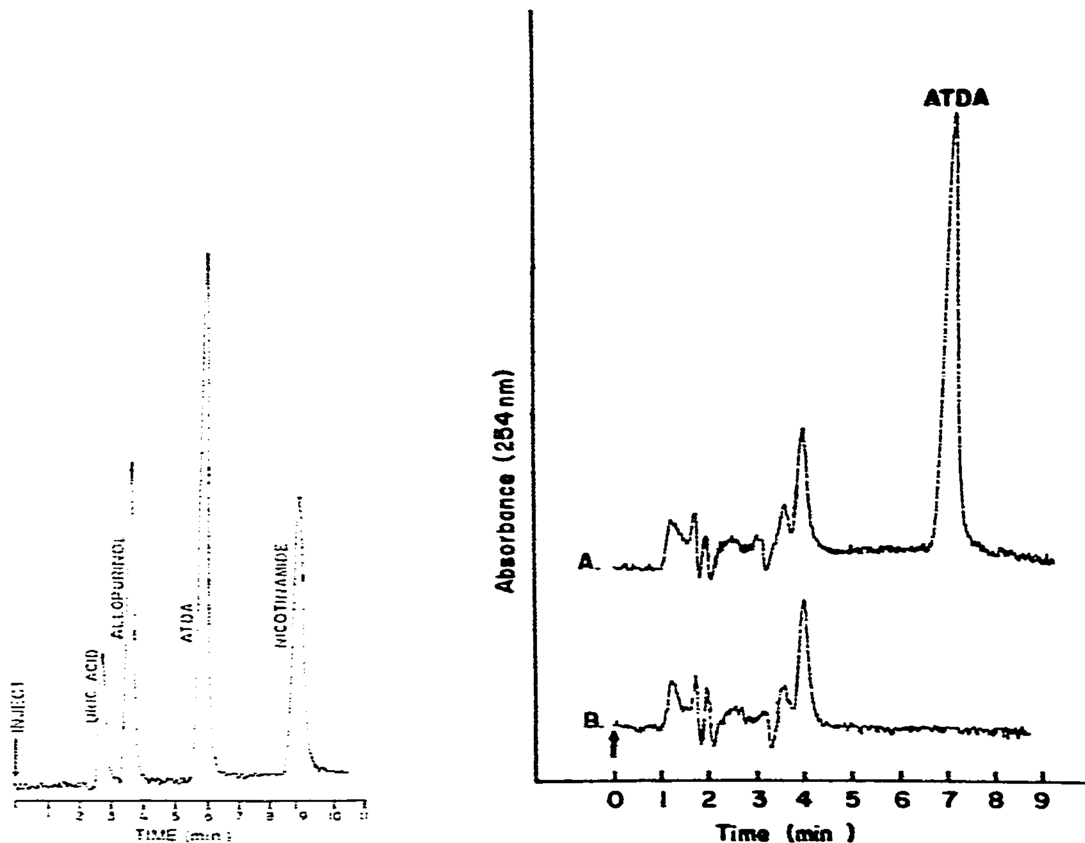

Fig. 2. Typical HPLC trace demonstrating separation of uric acid, allopurinol, ATDA, and nicotinamide. A single stock solution containing all four compounds was prepared. A 100-μl aliquot (containing 250 μg of each compound) was then injected manually onto the column. Separation of the compounds was achieved as described in Experimental.

Fig. 3. Representative chromatograms of TLC-processed serum samples obtained from a patient 5 min after administration of 125 mg/m^2 ATDA (A) and a pre-drug administration control serum sample (B). Peaks eluting prior to 4 min were not identified. Arrow indicates point of injection.

recovery of ATDA from human serum. Unfortunately, an internal standard was not feasible as there are not any established metabolites or close structural analogues of ATDA readily available. Inter-assay variability of ATDA standard proved to have a coefficient of variation of less than 2.5% (n = 17).

No retention of ATDA was observed on a reversed-phase column using simple mobile phases of water and methanol. The use of 1-octanesulfonic acid as a paired-ion reagent in the acidic mobile phase resulted in sufficient retention for accurate separation and quantitation. Pic B-8 reagent is buffered to a pH of 3.0 with acetate and when mixed with the mobile phase described, results in a constant pH of 3.0. The relative size of the lipophilic group on the counter ion affected the degree of retention of ATDA on the C_{18} reversed-phase column. Retention time of ATDA was observed to decrease when the paired-ion reagent 1-octanesulfonic acid was changed to 1-pentanesulfonic acid. This phenomenon has also been described for drugs other than ATDA [6].

This method satisfactorily determines ATDA in the presence of such potentially interfering agents as nicotinamide, uric acid and allopurinol. Allopurinol is oxidized in vivo to the major metabolite alloxanthine (oxopurinol) [7]. Comparing the levels of ATDA and the elimination half-life, which are essentially identical for patients who received allopurinol and for those who did not, clearly demonstrates that neither allopurinol nor alloxanthine interferes with ATDA analysis.

To the best of our knowledge the present report represents the first method for the routine separation and quantitation of nonradioactive ATDA from biological fluids. El Dareer et al. [4] have recently reported the distribution and metabolism of [C^{14}] ATDA in mice, dogs, and monkeys using a TLC system. They have reported that in mice injected intraperitoneally with 100 mg/kg of ATDA, serum drug levels decrease with a half-life of 2.9 h. We have obtained nearly identical results (half-life 2.2 h) using nonradioactive ATDA and the TLC—HPLC method described here (Fig. 4). In addition, we report preliminary data on the kinetics of ATDA in humans. As seen in Fig. 4 the serum disappearance curve for ATDA in humans closely resembles that observed in mice. It merits emphasis that the extrapolated C_0 levels of ATDA that we estimate in mice (90 μg/ml) are very close to those (100—110 μg/ml) reported by El Dareer et al. [4] and roughly comparable (considering the difference in dose) to that recently reported by Lu et al. [5] in dogs.

The drug elimination half-life reported in the present study was determined from the data which were subjected to least-squares regression analysis. More extensive studies are obviously required for complete kinetic analyses and for

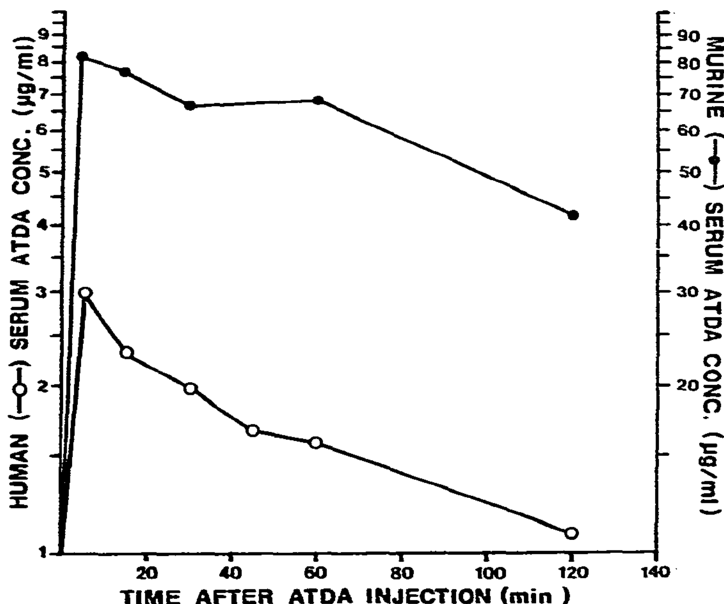

Fig. 4. Mean serum drug concentrations from six patients administered 50 mg/m² and from mice administered 300 mg/m² ATDA (4 mice per time point). ATDA levels were determined as described in Experimental.

an investigation into possible urinary metabolites such as those reported by El Dareer et al. [4]. We are currently applying this TLC—HPLC method for analysis of ATDA to more extensive studies of the pharmacokinetics of this oncolytic agent. In principle this method should be applicable to analysis of related compounds, such as 2-ethylamino-1,3,4-thiadiazole, an analogue of ATDA that recently was reported to exert beneficial effects in an experimental model of circulatory shock [8].

ACKNOWLEDGEMENTS

This work was supported by grants CA 22435, CA 24543 and CA 09324 and contract No. NO1 CM 97278 awarded by the National Cancer Institute.

The authors wish to thank Linda Mathews and Joan Braddock for skillful technical assistance.

REFERENCES

1 I.H. Krakoff and G.B. Magill, Proc. Soc. Exp. Biol. Med., 91 (1956) 470.
2 I.H. Krakoff and M.E. Balis, J. Clin. Invest., 38 (1959) 907.
3 I.H. Krakoff and M.E. Balis, Ann. Pharm. Dis., 25 (1966) 651.
4 S.M. El Dareer, K.F. Tillery and D.L. Hill, Cancer Treat. Rep., 62 (1978) 75.
5 K. Lu, J.P. Chang and T.L. Loo, Proc. Amer. Assoc. Cancer Res., 18 (1977) 169.
6 J. Korpi, D.P. Wittmer, B.J. Sandmann and W.G. Haney, Jr., J. Pharm. Sci., 65 (1976) 1087.
7 G.B. Elion, Handb. Exp. Pharmakol. 51 (1978) 485.
8 J. Abraham, R.W. Hopkins, C.A. Damewood and F.A. Simeone, Circ. Shock, 7 (1980) 373.